

Two Host-Induced *Ralstonia solanacearum* Genes, *acrA* and *dinF*, Encode Multidrug Efflux Pumps and Contribute to Bacterial Wilt Virulence^{∇†}

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Multidrug efflux pumps (MDRs) are hypothesized to protect pathogenic bacteria from toxic host defense compounds. We created mutations in the *Ralstonia solanacearum* *acrA* and *dinF* genes, which encode putative MDRs in the broad-host-range plant pathogen. Both mutations reduced the ability of *R. solanacearum* to grow in the presence of various toxic compounds, including antibiotics, phytoalexins, and detergents. Both *acrAB* and *dinF* mutants were significantly less virulent on the tomato plant than the wild-type strain. Complementation restored near-wild-type levels of virulence to both mutants. Addition of either *dinF* or *acrAB* to *Escherichia coli* MDR mutants KAM3 and KAM32 restored the resistance of these strains to several toxins, demonstrating that the *R. solanacearum* genes can function heterologously to complement known MDR mutations. Toxic and DNA-damaging compounds induced expression of *acrA* and *dinF*, as did growth in both susceptible and resistant tomato plants. Carbon limitation also increased expression of *acrA* and *dinF*, while the stress-related sigma factor RpoS was required at a high cell density ($>10^7$ CFU/ml) to obtain wild-type levels of *acrA* expression both in minimal medium and in planta. The type III secretion system regulator HrpB negatively regulated *dinF* expression in culture at high cell densities. Together, these results show that *acrAB* and *dinF* encode MDRs in *R. solanacearum* and that they contribute to the overall aggressiveness of this phytopathogen, probably by protecting the bacterium from the toxic effects of host antimicrobial compounds.

Many bacteria can survive and even grow in the presence of toxic compounds (48). One of the means by which bacteria survive in toxic environments is by extruding toxins through membrane-bound efflux pumps (7, 69). These efflux proteins, called multidrug resistance efflux pumps (MDRs), transport a broad range of structurally unrelated compounds out of the cell and can confer resistance to a wide variety of toxins, including antibiotics (7, 36).

The following five MDR families have been characterized: (i) the ATP binding cassette (ABC) superfamily (13), (ii) the major facilitator superfamily (54, 55, 66), (iii) the resistance nodulation-cell division (RND) superfamily (66), (iv) the small multidrug resistance (SMR) superfamily (47), and (v) the multidrug and toxic compound extrusion (MATE) superfamily (10). These superfamilies vary in the mechanism of transport, the number of transmembrane domains, and substrate specificity. The SMR superfamily has been found only in prokaryotes, while members of the RND, major facilitator, ABC, and MATE superfamilies are present in all domains of life (48).

The role of MDRs in human and animal pathogens in association with the emergence of antibiotic-resistant strains has been well studied (36). However, comparative genomic analy-

ses have revealed that MDRs are widely distributed in both pathogenic and nonpathogenic bacteria (55). This ubiquity underscores the importance of MDRs in bacterial life cycles. Despite the genomic abundance of apparent MDR-encoding genes, we have only begun to understand the contribution of MDRs to plant-pathogenic and environmental bacteria.

Plants respond to microbial attack with sophisticated defenses that include the production of antimicrobial peptides and secondary metabolites, such as flavonoids, isoprenoids, and alkaloids (19, 45, 57). It has been hypothesized that plant pathogens must neutralize the toxicity of these compounds to succeed in the hostile host environment (67). In fact, MDRs contribute significantly to the virulence of several plant-associated fungi, including *Botrytis cinerea*, *Gibberella pulcaris*, and *Mycosphaerella graminicola* (18, 59, 71).

Recent work has demonstrated that efflux pumps in the plant-associated bacteria *Pseudomonas syringae*, *Agrobacterium tumefaciens*, *Rhizobium etli*, and *Bradyrhizobium japonicum* are important for establishing a successful plant-bacterium interaction, but the specific role of MDRs in these interactions is still unclear (23, 32, 35, 46, 50). In addition, MDRs also contribute to the pathogenic fitness of enterobacterial phytopathogens. For example, *Erwinia amylovora*, which causes fire blight of apples and pears, requires AcrAB (a member of the RND superfamily) for successful colonization and pathogenesis in apple rootstock (12). Another recently characterized MDR in *E. amylovora*, NorM (a MATE superfamily member), is essential for successful competition with other bacteria in the same niche (11). In the soft rot pathogen *Erwinia chrysanthemi*, inactivation of *tolC*, which encodes the outer membrane protein of the tripartite AcrAB-TolC pump, impaired the ability of the bacterium to macerate endive leaves and to grow in planta.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics ^a	Reference or source
<i>E. coli</i> strains		
DH5 α	F ⁻ <i>endA1 relA</i> ϕ 80 <i>lacZ</i> Δ M15 <i>hsdR17 supE44 thi-1 recA1 gyrA96</i> Nal ^r	24
KAM3	Δ <i>acrA</i> strain derived from <i>E. coli</i> TG1	40
KAM32	Δ <i>acrA</i> Δ <i>yhge</i> strain derived from <i>E. coli</i> TG1	30
<i>R. solanacearum</i> strains		
K60	Wild-type race 1 biovar 1 tomato isolate	33
KDF	K60 <i>dinF</i> ::Gm ^r	This study
K1833	K60 <i>acrA</i> ::Gm ^r	This study
K60-phcA	K60 <i>phcA</i> :: Ω , Sm ^r	1
K999	K60 <i>rpoS</i> Km ^r	9
K200	K60 <i>hrpB</i> ::Gm ^r	51
Plasmids		
pBluescript SK(-)	Ap ^r	Stratagene
pLAFR3	Tc ^r , replicates in <i>R. solanacearum</i>	61
PLAFacRA	<i>acrA</i> -containing cosmid (7-43), Tc ^r	This study
PLAFdinF	<i>dinF</i> -containing cosmid (6-43), Tc ^r	This study
pSTBlue-1	AT cloning vector, Ap ^r Km ^r	Novagen, Inc.
pSTacRA SOE	SOE PCR product with introduced BglIII restriction site	This study
pSTacRA::Gm	<i>acrA</i> ORF disrupted by Gm ^r cassette	This study
pSTdinF::Gm	<i>dinF</i> ORF disrupted by Gm ^r cassette	This study
pUCGm	Gentamicin resistance cassette (<i>aacC1</i>)	60
pSTacRA	<i>acrA</i> ORF in pSTBlue-1	This study
pSTdinF	<i>dinF</i> ORF in pSTBlue-1	This study
pBSdinF	<i>dinF</i> ORF in pBluescript	This study
pVO155	Promoter probe plasmid with promoterless <i>uidA</i> gene, Km ^r	44
pDB155	Promoter probe plasmid with promoterless <i>uidA</i> gene, derived from pVO155, Sm ^r	9
pVOdinF	<i>dinF</i> promoter and 5' region (600 bp) in pVO155	This study
pDBdinF	<i>dinF</i> promoter and 5' region (600 bp) in pDB155	This study
pVOacrA	<i>acrA</i> promoter and 5' region (1,200 bp) in pVO155	This study
pDBacrA	<i>acrA</i> promoter and 5' region (1,200 bp) in pDB155	This study
pPilA-FOR	<i>pilA</i> promoter and 5' region in pVO155 in forward orientation	9

^a Ap, ampicillin; Tc, tetracycline; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Nal, nalidixic acid.

The TolC mutant also exhibited increased sensitivity to toxic compounds from a variety of plant species (5).

Ralstonia solanacearum is a gram-negative soilborne phytopathogen that has an unusually wide host range, and it causes bacterial wilt disease on important food crops, such as banana, tomato, and potato (26). This pathogen enters the host through wounded roots and rapidly colonizes the xylem vessels, reaching densities of 10¹⁰ CFU/ml of xylem fluid. The leaves of plants infected with *R. solanacearum* wilt due to impaired water transport; once wilt symptoms appear, the infected plant quickly succumbs to the bacterium. The high incidence of plant mortality, coupled with the scarcity of effective control methods, makes *R. solanacearum* one of the world's most destructive bacterial plant pathogens (26, 52). The genome sequences of two *R. solanacearum* strains (20, 56) have facilitated molecular analysis of virulence in this pathogen. To date, studies have demonstrated that *R. solanacearum* virulence is complex and depends on diverse, environmentally regulated, quantitative virulence factors, including extracellular polysaccharide, extracellular enzymes, and the cumulative effects of multiple type III secretion system-dependent effectors (21, 58).

Previously, we performed an in vivo expression technology screen for *R. solanacearum*, which identified 153 genes that are up-regulated during growth and pathogenesis in tomato (9). The following two genes encoding putative MDRs were induced in plants: *acrA* and *dinF* (encoding DNA damage-induc-

ible protein F, a member of the MATE superfamily). Because two MDRs belonging to distinct protein families are upregulated, we hypothesized that *R. solanacearum* must actively expel diverse toxic compounds to successfully cause disease in tomato. To test this hypothesis, we cloned and mutated the genes encoding these two MDRs (*acrA* and *dinF*). Here we describe characterization of the *acrA*- and *dinF*-encoded MDRs, their regulation, and their role in bacterial wilt virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *R. solanacearum* mutant strains used in this study were derived from wild-type race 1 biovar 1 strain K60 (33). *R. solanacearum* cells were grown at 28°C in CPG broth (27) or on CPG agar plates supplemented with 0.05% (wt/vol) 2,3,5-triphenyltetrazolium chloride (33). Boucher's minimal medium (BMM) (8) was supplemented with 0.2% glucose when minimal medium was required. When needed, antibiotics were added to the media at the following final concentrations: 25 to 50 mg/liter kanamycin, 12.5 to 25 mg/liter gentamicin, 30 mg/liter streptomycin, 100 mg/liter ampicillin, and 20 mg/liter nalidixic acid. The growth rates of wild-type and mutant strains in BMM broth amended with 0.2% (wt/vol) glucose, in CPG broth, and in tobacco leaf tissue (cultivar Bottom Special) were compared as previously described (63). *Escherichia coli* strains were grown in Luria-Bertani or Mueller-Hinton broth supplemented with the appropriate antibiotic (4). The bacterial strains and plasmids used in this study are shown in Table 1.

DNA manipulations. Isolation of plasmid and chromosomal DNA, restriction site analysis, cloning, and PCR were performed using standard methods (4). *R. solanacearum* and *E. coli* were transformed by electroporation as previously

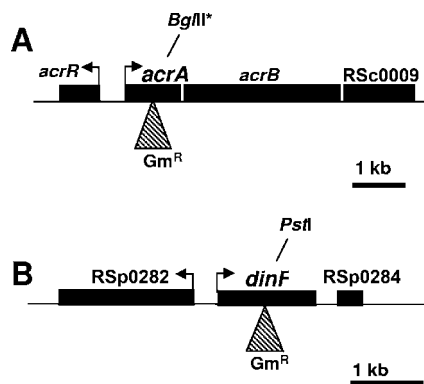


FIG. 1. Generation of *R. solanacearum* *acrA* and *dinF* mutants. Mutations were created by inserting a gentamicin resistance cassette (Gm^R) into the ORF of either *acrA* (A) or *dinF* (B), as indicated. The arrows indicate the directions of transcription. Flanking genes are included for reference and are annotated as follows: RSc0009, encoding a drug efflux lipoprotein (NodT/FusA family); RSp0282, encoding a hypothetical amino acid permease; and RSp0284, encoding a hypothetical transmembrane protein. The asterisk indicates that the BglII site was introduced.

described (1). DNA sequencing was performed at the University of Wisconsin-Madison Biotechnology Center, Madison. Oligonucleotides were obtained from Sigma Genosys (The Woodlands, TX). The DNA sequence analysis was performed using Biology Workbench (<http://workbench.sdsc.edu/>), NCBI (<http://www.ncbi.nlm.nih.gov/>), and NEB cutter (www.neb.com), and the GMI1000 genomic database (<http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/ralsto/index.html>) was also used.

Cloning, mutagenesis, and complementation of *acrA* and *dinF*. To mutate the *acrA* gene of *R. solanacearum*, an approximately 1,200-bp fragment containing the *acrA* open reading frame (ORF) was PCR amplified using primers 5'-CCG TTATCACCACCTCGCTG3' and 5'-GTCGACGATCACCTTGTCAC3' designed by using the GMI1000 sequence. The resulting PCR product was AT cloned into pSTBlue-1 (Novagen) to create pSTacrA. Splicing by overlap (SOE) PCR (29) was used to introduce a unique BglII site into pSTacrA. For the SOE PCR experiment, primers were designed to amplify the 5' and 3' halves of the cloned *acrA* fragment in pSTacrA, such that a BglII site was introduced into the center of the *acrA* ORF. The final SOE PCR product was AT cloned into pSTBlue-1 (Km^r ; Novagen) to obtain pSTacrASOE. The primers used for the SOE PCR included the forward and reverse primers described above, as well as 5'-GGAGATCTCTCCTAGAAAATGGGCGAGGTGACGGT3' and 5'-TCTA GAGGAAGATCTTCAATCTACCTGACCTTCACGCA3' (the BglII site is underlined). The gentamicin resistance cassette *aacC1* from pUCGM (60) was inserted into the introduced BglII site. The *acrA::Gm^r* construct pSTacrA::GM was introduced into *R. solanacearum* by electroporation, and double recombinants (Gm^r Km^r) were selected. Correct insertion of the *acrA* mutagenesis construct was confirmed by Southern blot hybridization analysis (data not shown). Since the *R. solanacearum* *acrABR* locus is very similar to the *E. coli* locus and *acrA* and *acrB* are separated by only 29 nucleotides, it is likely that the two genes are cotranscribed. Also, since the proteins must function together, a mutation in *acrA* and a mutation in *acrB* should result in the same phenotype.

To create a *DinF* mutant, a 2.1-kb fragment containing the entire *dinF* ORF was PCR amplified using primers designed by using the GMI1000 database (5'-AGCATCGACCACTTGG3' and 5'-ATGTGCAGCATCCAC3'). The PCR product was AT cloned into pSTBlue-1 (Km^r ; Novagen) to create pSTDinF. The 2.1-kb fragment containing the *dinF* gene was cloned into pBluescript (Stratagene) to obtain pBSDinF. The *aacC1* (Gm^r) gene cassette was introduced into the *dinF* ORF at a unique PstI site in pBSDinF to obtain pDinF::Gm, which was introduced into *R. solanacearum* by electroporation. Cells were selected on agar plates containing gentamicin and screened for double recombination on agar plates containing double-strength ampicillin. Correct insertion of the *dinF* mutagenesis construct was confirmed by Southern blot hybridization analysis (data not shown).

For in planta complementation, we used cosmids pLAFacrA and pLAFdinF, which contained the full-length *acrA* and *dinF* genes, respectively (as well as flanking sequences) (Fig. 1). These cosmids were obtained by screening the *R.*

solanacearum strain K60 genomic library by Southern hybridization using the *acrA* and *dinF* PCR products as probes. Cosmids harboring the *acrA* and *dinF* loci were introduced into the corresponding mutant strains by electroporation. Plant assays to confirm restoration of the mutant phenotypes to the wild-type phenotype were performed as described below.

Construction of GUS reporter strains. For transcription expression analysis of *dinF* and *acrAB* using β -glucuronidase (GUS) as a reporter, we inserted the 5' regions of both the *acrA* and *dinF* ORFs upstream of the promoterless *uidA* gene (encoding GUS) into plasmid pVO155 (Km^r) or pDB155 (Sm^r) (9). The *dinF::uidA* reporter construct was created by cloning a 600-bp EcoRI/PstI fragment from pSTDinF into pBluescript (Amp^r; Stratagene) to create pBSDinF. The promoter region and 5' end of *dinF* were removed from pBSDinF using XbaI and XhoI and directionally cloned into both pVO155 and pDB155 upstream of the promoterless *uidA* genes in these vectors to create pVOdinF and pDBdinF, respectively. The *acrA::uidA* reporter construct was created by cloning a 1,200-bp BamHI/XbaI fragment (containing approximately 300 bp of upstream sequence and most of the *acrA* ORF) from pSTacrA into pVO155 and pDB155, which were digested with BamHI and SpeI to create pVOacrA and pDBacrA. The resulting GUS reporter constructs were introduced by triparental conjugation into the following *R. solanacearum* strains: K60 (wild type), K200 (*hrpB*) (51), and K999 (*rpoS*) (9). Since neither pVO155 nor pDB155 could replicate in *R. solanacearum* under antibiotic selection conditions, integration into the chromosome must have occurred at the site of homology to the cloned gene fragment. This integration introduced a single copy of the promoterless GUS reporter gene, *uidA*, and created a *cis* merodiploid of the gene of interest, leaving an intact functional copy of the gene.

Determination of MICs. The MICs of various compounds were determined by a standard twofold serial dilution method (12). For *R. solanacearum* strains, MIC assays were carried out using BMM supplemented with 0.2% glucose containing different compounds at various concentrations. For *E. coli* MDR mutant strains KAM3 and KAM32, MIC assays were performed using Mueller-Hinton broth (Becton-Dickinson, Germany). MIC assays were performed in triplicate by using final volumes of 3 ml for *R. solanacearum* and 200 μ l for KAM3 and KAM32. The MIC corresponded to the concentration in the tube that showed no growth, as determined by comparison to an uninoculated control both visually and spectrophotometrically (absorbance at 600 nm). When appropriate, controls containing only the drug solvent were included in the MIC determinations to ensure that the solvent did not affect bacterial growth.

GUS assays. The in planta GUS assays were performed as described by Brown and Allen (9). Briefly, *R. solanacearum* cells harboring the reporter gene constructs were grown overnight in CPG medium, washed twice with sterile deionized water, and diluted to obtain an optical density at 600 nm of 0.1 (corresponding to 1×10^8 CFU/ml). Two-microliter portions of each diluted bacterial suspension were applied to cut petioles of 23-day-old susceptible (cultivar Bonny Best) or resistant (cultivar Hawaii 7996 [H7996]) tomato plants in order to deliver $\sim 10^5$ CFU to each petiole. Bacteria were harvested from a pool of six inoculated plants at several times (to obtain bacteria at different cell densities) by removing each infected petiole and 0.5 cm of the stem above and below the petiole, weighing the plant material, and then crushing the infected tissue in a mortar and pestle with 500 μ l of water. A 50- μ l sample was removed for enumeration of bacteria by dilution plating, and 450 μ l of GUS extraction buffer (31) was added to the ground tomato tissue, which was then stored at -80°C until it was used for the GUS assays. To allow comparisons of GUS activity in planta and in culture, the number of CFU per gram of plant tissue was multiplied by 13.75 to obtain the equivalent number of CFU per milliliter of xylem fluid, as previously described (64). Strains carrying the integrated, constitutively expressed *pilA* promoter region cloned into pIVETDGB (a derivative of pVO155) were used as a positive control for GUS expression (data not shown). For the in planta GUS assays, 25-day-old cultivar Bonny Best and H7996 seedlings (11 days after transplanting) were used. Each assay was repeated three times.

For GUS assays in culture, bacteria were grown overnight in CPG medium, washed with sterile deionized water, and then resuspended in BMM (supplemented with 0.2% glucose unless indicated otherwise) to obtain an optical density at 600 nm of 1.0 (corresponding to 1×10^9 CFU/ml). The bacterial suspension was diluted to obtain various cell densities in BMM containing 0.2% glucose and incubated on an environmental shaker at 28°C at 250 rpm for 6 h (about ~ 1.5 bacterial generations). After this incubation, 100- μ l samples were removed to determine the cell density. Bacteria were collected by centrifugation (6,000 $\times g$) for 3 to 30 min (depending on the culture volume), and cell pellets were stored at -80°C until they were used for GUS assays.

To assess whether *dinF* or *acrA* expression is induced by toxic compounds, K60 cells harboring either the *dinF::uidA* or *acrA::uidA* GUS reporter constructs were resuspended to obtain a final cell density of 1×10^8 CFU/ml in BMM supple-

mented with toxic compounds at sublethal concentrations, as determined by MIC analysis as described above. Each bacterial suspension was incubated at 28°C with shaking (250 rpm) for 6 h; after incubation, the bacteria were harvested by centrifugation and stored at -80°C until they were used for measurement of GUS activity.

Measurement of GUS activity. Cells were permeabilized with lysozyme (200 µg/ml, 37°C, 20 min) in 1 ml of GUS extraction buffer, and GUS activity was assayed with the substrate methylumbelliferyl-glucuronide as previously described (31, 64). The level of fluorescence of the released methylumbelliferone (MU) was determined using a Hoefer fluorometer calibrated with a known concentration of MU. GUS activity was expressed in nanomoles of MU produced per minute per cell and was graphed against the cell density (expressed in CFU/ml) in either liquid culture or tomato xylem fluid.

Plant growth conditions and assays. The susceptible heirloom tomato cultivar Bonny Best and the resistant cultivar H7996 (25) were grown in a growth chamber at 28 to 30°C by using a cycle consisting of 12 h of light and 12 h of darkness. Sixteen-day-old Bonny Best plants were used for soil soak virulence assays as previously described (63). Briefly, 50 ml of a bacterial suspension containing $\sim 5 \times 10^7$ CFU/ml was poured onto tomato seedlings in 80 g of soil, resulting in a concentration of $\sim 3.1 \times 10^7$ CFU/g of soil. Diseased plants were rated daily using a disease index scale ranging from 0 to 4 as previously described (63). Plant assays were repeated at least three times, using 16 plants per treatment in each assay.

RESULTS

In *E. coli*, *acrA* encodes the periplasmic subunit of the tripartite AcrAB-TolC drug efflux pump, a member of the RND MDR superfamily (37). The *R. solanacearum* AcrA protein sequence (NCBI accession number NP 518132.1) was 71% similar to the *E. coli* AcrA protein sequence. Also, the genomic organization of the *acrAB* operon and the adjacent *acrR* locus (encoding the transcriptional repressor of the *acrAB* operon) was conserved in *E. coli* and *R. solanacearum*. The only difference between the *R. solanacearum* and *E. coli* *acrABR* loci was that *R. solanacearum* has a gene encoding a putative outer membrane channel belonging to the NodT/FusA family (RSc0009) that is present at or near the end of the *acrAB* operon (Fig. 1A), while *E. coli* lacks a gene that encodes an outer membrane channel at this locus (70).

DinF belongs to a distinct branch of the MATE superfamily of MDRs (10). BLASTP analysis showed that the *R. solanacearum* DinF protein (NCBI accession number NP 521844.1) resembles DinF homologs in *E. coli* (58% similarity) and *Streptococcus pneumoniae* (35% similarity). Yet the genomic synteny of *dinF*-flanking regions in *R. solanacearum* and other bacteria was not conserved (data not shown). In *E. coli* and *S. pneumoniae*, *dinF* is located in operons with *lexA* and *recA*, respectively (34, 41). However, in *R. solanacearum*, *dinF* is located on the organism's 2.1-Mb megaplasmid and is flanked by genes encoding a hypothetical amino acid permease (RSp0282) and a putative transmembrane protein (RSp0284) (Fig. 1B), while both *lexA* and *recA* are on the 3.7-Mb chromosome in *R. solanacearum*. Two highly conserved motifs, NIILDPLFI and GAAIATVIA, which are characteristic of the DinF/VmrA cluster of the MATE superfamily (10, 30), are present in the *R. solanacearum* DinF protein sequence, as follows: NMVAVLGLV and GIGAATAVA (conserved and similar residues are underlined and in boldface type, respectively). The presence of these conserved residues suggested that *R. solanacearum* DinF is a member of the DinF branch of the MATE superfamily rather than the NorM branch, which lacks this conserved sequence.

To investigate the overall relationship between the *R. so-*

lanacearum AcrA and DinF proteins and homologues in other bacteria, we performed a ClustalW analysis, and a phylogenetic tree was generated using Tree puzzle 5.2 (www.tree-puzzle.de) for 35 MATE superfamily sequences and 28 AcrA sequences derived from a BLASTP search (2) (see Fig. S1 and S2 in the supplemental material). The AcrA protein sequence was highly conserved in all bacterial strains tested. However, the *R. solanacearum* DinF protein sequence was less similar to the sequences of other DinF homologs and MATE superfamily proteins (including four NorM sequences and two *Arabidopsis thaliana* MATE superfamily proteins). Importantly, the *R. solanacearum* DinF sequence clustered with the sequences of other proteins in the DinF branch of the MATE superfamily. Also, the DinF and AcrA proteins from *R. solanacearum* clustered in the same branch as orthologues from other phylogenetically related proteobacteria, such as *Burkholderia cepacia* (DinF) and *Ralstonia metallidurans* (AcrA).

We created site-directed mutants with the K60 wild-type background, including K1833 (*acrAB*) and KDF (*dinF*) (see Materials and Methods) (Fig. 1). Both K1833 and KDF grew at wild-type levels in minimal medium supplemented with 0.2% glucose, indicating that they had no general in vitro growth defect and were not auxotrophs. To ensure that the loss of DinF or AcrA did not prevent *R. solanacearum* from multiplying in plant tissue, which is a prerequisite for virulence in this pathogen, we also measured the multiplication of both mutants following infusion into leaves of susceptible tobacco cultivar Bottom Special. This tobacco assay is a rapid and reliable test for the ability of *R. solanacearum* cells to multiply in planta but is not a means by which pathogenicity can be tested since the bacteria are injected into the leaf apoplast, which is not the usual niche for *R. solanacearum* in the plant. In contrast, the numbers of pathogen cells in tomato xylem reflect disease progress and are correlated with virulence, as well as the general fitness in planta. The growth of K1833 and KDF in tobacco leaves was indistinguishable from the growth of the wild-type parent strain (data not shown).

To measure the contributions of *acrAB* and *dinF* to *R. solanacearum* virulence, we performed a soil inoculation assay in which bacterial suspensions were poured into pots containing unwounded susceptible tomato seedlings. This inoculation method mimics the natural *R. solanacearum* infection process, where the bacteria must locate and enter the roots from the soil. Both K1833 and KDF were significantly attenuated for the ability to cause wilt symptoms in tomato compared to wild-type strain K60 (Fig. 2).

The virulence phenotypes of K1833 and KDF were complemented by introducing cosmids (with the pLAFR3 vector backbone) that harbored the full-length *acrAB* operon and the complete *dinF* ORF, respectively, each flanked by at least 1 kb of DNA on each side of the locus. For both K1833 and KDF, addition of the cosmids restored virulence to the mutant strains (Fig. 2).

MDR mutants of other plant-pathogenic bacteria are less resistant to various toxins (5, 11, 12, 50). Therefore, we determined the relative MICs of several toxic antibiotics, phytoalexins, and detergents for K1833 and KDF in minimal medium (Table 2). As expected, both mutants exhibited low but measurable sensitivities to various toxic compounds, including caffeic acid and tomatine, which are antimicrobials produced by

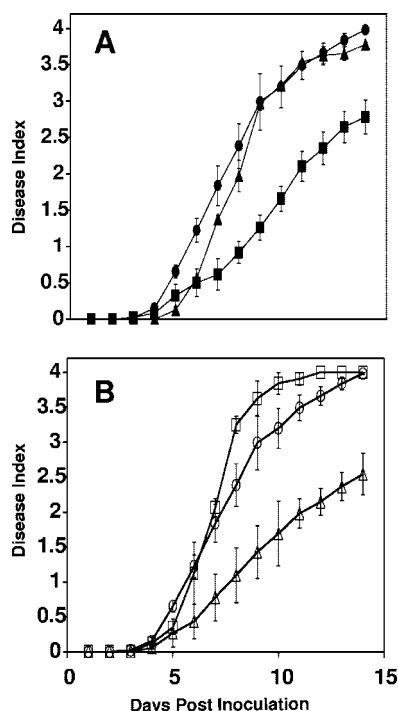


FIG. 2. Virulence of *R. solanacearum* *acrA* and *dinF* strains on susceptible tomato cultivar Bonny Best is significantly reduced. The curves are disease progress curves for *R. solanacearum* *acrA* (A) and *dinF* (B) mutant strains. A bacterial suspension was added to the soil of 14-day-old tomato seedlings to obtain a bacterial density of approximately 3.1×10^7 CFU/g of soil, and plants were rated on a disease index scale ranging from 0 to 4. (A) ●, wild-type strain K60; ■, *acrA* strain K1833; ▲, complemented strain K1833(pLAFacrA). (B) ○, wild-type strain K60; △, *dinF* strain KDF; □, complemented strain KDF(pLAFdinF). The symbols indicate the averages for six plant assays, each performed with 16 plants per treatment; the error bars indicate the standard errors of the means.

tomato plants. Neither mutant exhibited increased sensitivity to biochanin A, gossypol, mitomycin C, plumbagin, pyrithione, rhein, or salicylic acid. *acrAB* mutant K1833 was sensitive to a greater number of compounds than KDF, while KDF was sensitive to only two compounds (4',6'-diamidino-2-phenylindole [DAPI] and tetraphenylphosphonium chloride [TPPCI]) to which K1833 was resistant. These results suggest that the protein products of *acrAB* and *dinF* have overlapping substrate specificities but that some compounds are specifically transported by only one MDR.

To verify the biological function of the *R. solanacearum* *dinF* and *acrAB* gene products, we examined whether either product could heterologously restore toxin resistance to the *E. coli* AcrAB deletion mutant KAM3 (40). A plasmid containing either *dinF* or *acrAB* was introduced to the *E. coli* mutant strain in *trans*, and the MICs of several toxic compounds for the resulting hybrid strain were determined (Table 3). The presence of *R. solanacearum* *acrAB* or *dinF* increased KAM3 resistance to ethidium bromide and acriflavine twofold and eightfold, respectively. The presence of *acrAB* also resulted in a twofold increase in resistance to berberine, while the presence of *dinF* resulted in a fourfold increase in resistance. *R. solanacearum* *dinF* also increased KAM3 resistance to nalidixic

TABLE 2. MICs of various compounds for *R. solanacearum* *acrA* and *dinF* mutant strains

Compound	MIC ^a		
	Wild-type strain K60	<i>acrA</i> strain	<i>dinF</i> strain
Acriflavine	62.50	15.60	15.60
Ampicillin	62.50	15.60	62.50
Berberine	62.50	15.60	62.50
Biochanin A	>1,000	>1,000	>1,000
Caffeic acid	250.0	125.0	250.0
Cephalexin	500.0	250.0	500.0
Chloramphenicol	31.30	15.60	31.30
DAPI	3.130	3.130	1.560
Esculetin	31.30	15.60	15.60
Ethidium bromide	62.50	7.813	31.30
Fusaric acid	125.0	31.30	125.0
Gossypol	>1,000	>1,000	>1,000
Hydrogen peroxide	3.125	3.125	2.081
Mitomycin C	25	25	25
Nalidixic acid	62.50	15.60	62.50
Plumbagin	62.50	62.50	62.50
Pyrithione	3.910	3.910	3.910
Resorcinol	250.0	62.50	250.0
Rhein	31.30	31.30	31.30
Salicylic acid	1,000	1,000	1,000
Sodium dodecyl sulfate	250.0	125.0	250.0
Tomatine ^b	>250	62.50	125.0
TPPCI	1,000	1,000	125.0

^a The MICs are the minimum concentrations that inhibited bacterial growth. Most MICs are expressed in $\mu\text{g/ml}$; the only exceptions are the MICs of hydrogen peroxide, which are expressed in mM. The assays were performed in triplicate, and the optical densities at 600 nm were normalized to 0.001 at the beginning of the MIC assay. MICs that differ from the MIC for the wild-type strain are indicated by boldface type.

^b For tomatine, BMM was buffered with 0.1 M morpholineethanesulfonic acid (MES) to pH 7.0 for the MIC assay.

acid and berberine twofold. There was no difference in the resistance of KAM3 to esculetin or TPPCI in the presence of either *R. solanacearum* protein. In addition, the presence of *dinF* in the KAM32 mutant (which lacked both AcrAB and the MATE superfamily protein YdhE, which we hypothesize functions like DinF) (14) doubled its resistance to TPPCI and berberine, quadrupled its resistance to acriflavine, increased its resistance to ampicillin eightfold, and increased its resistance to ethidium bromide 16-fold (Table 3). We examined the effect

TABLE 3. MICs for *E. coli* strains KAM3 and KAM32 harboring *acrA* or *dinF* from *R. solanacearum*

Compound	MIC (mg/ml) ^a				
	KAM3 ^b	KAM3 (pLAFacrA)	KAM3 (pLAFdinF)	KAM32 ^b	KAM32 (pLAFdinF)
Acriflavine	0.49	3.9	3.9	1.9	7.8
Ampicillin	3.9	7.8	3.9	3.9	31.3
Berberine	31.3	62.5	125.0	15.6	31.3
Esculetin	500	500	500	ND ^c	ND
Ethidium bromide	3.9	7.8	7.8	0.95	15.6
Nalidixic acid	1.9	1.9	3.9	ND	ND
TPPCI	3.9	3.9	3.9	15.6	31.3

^a The MICs are the minimum concentrations required to inhibit bacterial growth. The assays were performed in triplicate to ensure consistent results. The optical densities at 600 nm were normalized to 0.001 at the beginning of the MIC assay. MICs that differ from the MICs for the parent mutant strains are indicated by boldface type.

^b KAM3 and KAM32 with empty plasmid pLAFR3 gave identical results.

^c ND, no data.

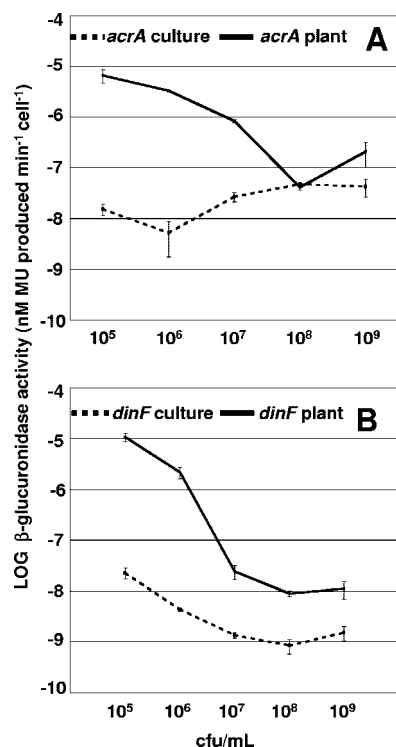


FIG. 3. *dinF* and *acrA* are highly expressed in planta. The GUS activities produced by strains carrying a *dinF::uidA* or *acrA::uidA* transcriptional fusion were measured for cells grown to different cell densities in planta (susceptible tomato) and in vitro (minimal broth). The cell density (expressed in CFU/ml of broth or xylem fluid) is indicated on the x axis. The data are the averages \pm standard errors of the means for three independent experiments performed with six plants per treatment. (A) *acrA* expression (in *R. solanacearum* strain K60 with pVOacrA); (B) *dinF* expression (in *R. solanacearum* strain K60 with pVODinF).

of adding *dinF* to KAM32 because this strain lacks both AcrAB and the MATE superfamily protein YdhE. This construct allowed us to more precisely determine the effect of heterologous expression of *dinF*, which is itself predicted to be a MATE superfamily protein. We included the *dinF* gene in the MIC tests for KAM3 in order to examine the functional redundancy between *dinF* and *acrAB*, but not in the MIC tests for KAM32 since it is unlikely that addition of *acrAB* to KAM32 would alter the MICs from those observed with KAM3.

To measure the expression of the *acrAB* and *dinF* genes in different environments, we created *cis* merodiploid reporter strains using a promoterless GUS gene (*uidA*) fused to the promoter and the 5' region of each gene (see Materials and Methods). The strains harboring the *acrA::uidA* and *dinF::uidA* transcriptional fusions were collected at various cell densities from cultures growing in minimal medium and in the xylem tissue of whole tomato plants. The expression of both *dinF* and *acrAB* increased more than 100-fold in the tomato xylem compared to the expression in minimal medium at low cell densities (10⁵ to 10⁶ CFU/ml) (Fig. 3). However, at higher cell densities, the differences in gene expression in BMM and in planta were smaller, suggesting that MDRs play a critical role during the initial stages of plant colonization by *R. solanacearum*. Expression of *dinF* varied as a function of cell density

both in planta and in culture, and the highest levels of expression were observed at lower cell densities. In contrast, *acrAB* expression was cell density dependent in both the plant and in minimal medium. Intriguingly, the expression in minimal medium increased about fivefold as the cell density increased, while the β -glucuronidase activity decreased with increasing cell density in planta until the cell density reached $\sim 10^8$ CFU/ml; then the expression started to increase.

The resistant tomato cultivar H7996 has been used to study *R. solanacearum* population dynamics in root and stem tissues (38, 43, 68). In H7996, *R. solanacearum* is largely confined to the primary xylem and poorly colonizes the root parenchyma cells. It has been hypothesized that the inability of *R. solanacearum* to effectively colonize H7996 is due to inhibitory compounds present in the resistant plants (43, 68). We reasoned that if H7996 produced toxins, then the expression of *acrAB* or *dinF* in *R. solanacearum* would increase during growth in this tomato cultivar. To test this possibility, we measured the GUS activities of both *acrA::uidA* and *dinF::uidA* fusion strains in H7996. We found that the expression of the *dinF::uidA* fusion doubled at low to moderate cell densities (10⁵ to 10⁸ CFU/ml) in H7996 compared to the expression in the susceptible tomato cultivar, while the expression of the *acrA::uidA* fusion was about 1.5-fold higher at similar cell densities compared to the expression in susceptible cultivar Bonny Best. However, at high cell densities (10⁹ CFU/ml), the relative levels of gene expression in H7996 decreased significantly (Table 4). These results showed that during the initial host colonization MDR gene expression was moderately increased in H7996 but at high cell densities the MDR gene expression in the resistant tomato cultivar was significantly lower than the MDR gene expression in the susceptible tomato cultivar.

We measured the relative levels of expression of *acrAB* or *dinF* in the presence and absence of sublethal concentrations of several toxins (Table 5). All of the compounds tested increased the expression of *acrAB* and *dinF* between 1.3- and 5.3-fold. The DNA-damaging toxin mitomycin C was the most potent inducer for both *acrAB* and *dinF*. The plant defense signaling molecule salicylic acid induced *acrAB* expression and *dinF* expression 1.3- and 2.1-fold, respectively. This is interest-

TABLE 4. *acrA* and *dinF* expression in resistant tomato cultivar H7996 compared to expression in susceptible tomato cultivar Bonny Best

Concn (CFU/ml)	Fold induction of GUS expression ^a	SE
<i>acrA::uidA</i> strain		
10 ⁵	1.511	0.247
10 ⁶	1.385	0.411
10 ⁸	1.807	0.329
10 ⁹	0.079	0.009
<i>dinF::uidA</i> strain		
10 ⁶	2.449	0.507
10 ⁸	2.104	0.001
10 ⁹	0.698	0.071

^a The fold induction was determined by dividing the GUS activity observed in resistant tomato cultivar H7996 by the expression in the susceptible tomato cultivar Bonny Best.

TABLE 5. Relative induction of *acrA* and *dinF* in the presence of various compounds^a

Compound ^b	Induction of:	
	<i>acrA</i>	<i>dinF</i>
Acriflavine	2.9 (0.03) ^c	1.6 (0.06)
Berberine	1.3 (0.09)	1.9 (0.02)
Esculetin	1.5 (0.02)	2.0 (0.03)
H ₂ O ₂	3.2 (0.02)	1.6 (0.17)
Mitomycin C	5.3 (0.07)	5.1 (0.10)
Salicylic acid	1.3 (0.01)	2.1 (0.03)
BMM alone	1.0	1.0

^a Relative induction was measured by determining GUS activity from *acrA::uidA* and *dinF::uidA* reporter gene fusions. The levels of expression are the levels relative to basal gene expression in minimal medium plus glucose (BMM).

^b Compounds were added at the following sublethal concentrations: acriflavine, 31.25 µg/ml; berberine, 31.25 µg/ml; esculetin, 16.25 µg/ml; H₂O₂, 5 mM; mitomycin C, 1.0 µg/ml; and salicylic acid, 500 µg/ml.

^c The data are means of three determinations; the values in parentheses are the standard errors.

ing since high concentrations of mitomycin C and salicylic acid did not inhibit the growth of either mutant (Table 2).

RpoS is an alternative sigma factor involved in bacterial stationary-phase and stress response pathways (28). Flaviv and coworkers (17) showed that the virulence of an *R. solanacearum* RpoS mutant was attenuated and that this mutant was unable to survive under carbon limitation conditions and at a low pH. Also, the RpoS mutant produced less of the quorum-sensing autoinducer acyl-homoserine lactone (17). To determine if RpoS is required for expression of *acrAB* in *R. solanacearum*, we introduced the *acrA::uidA* reporter fusion into *rpoS* mutant strain K999 (9) and measured *acrAB* expression during growth in minimal medium and in planta. We found that an intact copy of *rpoS* was necessary for wild-type levels of *acrAB* expression at cell densities greater than 10⁷ CFU/ml (Fig. 4). The same pattern of *rpoS*-dependent *acrAB* expression at high cell densities was observed in minimal me-

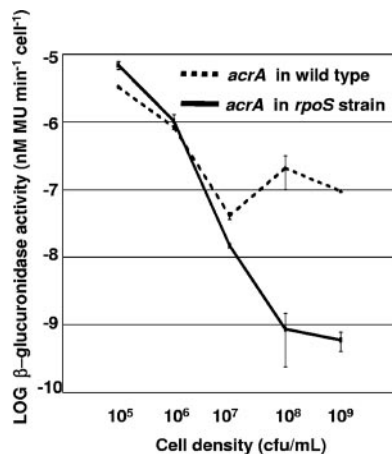


FIG. 4. RpoS is required for full *acrA* expression in planta at a high cell density. GUS activity in cells grown in the susceptible tomato cultivar Bonny Best was determined. The *acrA::uidA* fusion was introduced into wild-type K60 cells and the *rpoS* mutant strain (K999). The adjusted cell density (CFU/ml of xylem fluid) is indicated on the x axis. The data are the means ± standard errors of the means for three independent measurements obtained using six plants per treatment.

TABLE 6. *acrA::uidA* expression in wild-type strain K60 and the *phcA* and *rpoS* mutant strains increased in the absence of a carbon source

Bacterial strain (plasmid)	GUS activity (fmol MU released per cell per min) in BMM with ^a :	
	Carbon source	No carbon source
Wild-type K60 (pVO <i>acrA</i>)	0.0474 (0.003390)	0.210 (0.05370)
<i>phcA</i> (pVO <i>acrA</i>)	0.0854 (0.010700)	1.60 (0.20300)
<i>rpoS</i> (pDB <i>acrA</i>)	0.0120 (0.000015)	0.023 (0.00035)

^a The carbon source added was 0.2% glucose. Assays were performed in triplicate, and the values in parentheses are standard errors. Significantly different values, as determined by Student's *t* test at a *P* value of <0.001 with a 95% confidence interval, are indicated by boldface type.

dium (data not shown). We did not measure the expression of the *dinF::uidA* fusion in the *rpoS* mutant because, as shown in Fig. 3, *dinF* was expressed at a low cell density and not during the stationary phase.

In *E. coli*, slow growth and carbon starvation increase *acrAB* expression (53). To determine whether *acrAB* expression was affected by the absence of carbon in *R. solanacearum*, we measured *acrA::uidA* expression at a cell density of approximately 10⁸ CFU/ml after 4 h of growth (which corresponded to one generation) in minimal medium containing either no glucose (carbon starvation) or 0.2% glucose. Expression of *acrAB* increased ~4-fold in the absence of glucose (Table 6). The expression of the *dinF::uidA* fusion also increased slightly under these conditions (data not shown). In a preliminary experiment in which we assessed the dependence of *acrAB* expression on seven regulators (9), we observed that in cells growing on minimal medium agar, expression of *acrAB* was affected by the stationary-phase sigma factor RpoS and by the global virulence gene regulator PhcA. Therefore, we also measured the carbon-dependent expression of *acrAB* in mutants lacking PhcA or RpoS. In medium lacking a carbon source, *acrAB* expression was ~19-fold higher in the *phcA* mutant strain and ~8-fold higher in the wild-type genetic background. The absence of RpoS resulted in a twofold increase in *acrAB* expression in medium lacking carbon (Table 6). Overall, these data suggest that PhcA inhibits *acrAB* expression, while RpoS stimulates *acrAB* expression.

Cunnac et al. reported that the promoter region of *dinF* in *R. solanacearum* strain GMI1000 contained an Hrp_{II} box (TTTCG N₁₆TTTCG), which is recognized by HrpB (15). HrpB is an AraC-type transcriptional activator required for expression of the type III secretion system, which delivers effector proteins to the host cell (3). An *hrpB* mutant is avirulent on susceptible tomatoes and does not produce a hypersensitive response in resistant hosts (22). To experimentally determine if *dinF* expression is indeed dependent on HrpB, we introduced the *dinF::uidA* reporter construct pVO*dinF* into an *hrpB* mutant of strain K60, K200 (51), and measured the relative GUS activity during growth in minimal medium. In the absence of HrpB, *dinF* expression increased 3.4-fold at a cell density of 10⁷ CFU/ml, 8.7-fold at a cell density of 10⁸ CFU/ml, and 6.1-fold at a cell density of 10⁹ CFU/ml. However, there was no difference in *dinF* expression in the *hrpB* mutant at lower cell densities. In planta, *dinF* expression was similar in both wild-type and *hrpB* strains at cell densities less than 5 × 10⁶ CFU/ml (data not

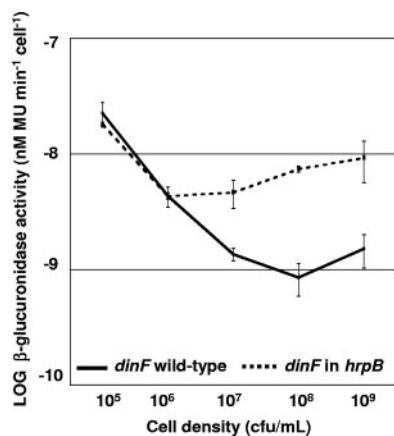


FIG. 5. *dinF* expression is increased in an *hrpB* strain at a high cell density. The GUS activity of the *dinF::uidA* fusion introduced into wild-type strain K60 and the *hrpB* mutant strain (K200) was measured using cells grown to different cell densities in BMM supplemented with 0.2% glucose. The cell density (CFU/ml) is indicated on the x axis. The data are the averages \pm standard errors of the means for three independent determinations.

shown); we were not able to measure the expression of *dinF* in the *hrpB* mutant at high cell densities in planta since this mutant cannot grow well in the tomato stem. Our results suggest that HrpB negatively regulates *dinF* expression (Fig. 5).

DISCUSSION

Putative toxin efflux pump genes are a prominent feature of the *R. solanacearum* genome, and we show here that two of these genes, *acrAB* and *dinF*, are upregulated during growth in host plants and that they are required for full virulence in susceptible tomato plants. Furthermore, both *acrAB* and *dinF* functioned heterologously as efflux pumps in toxin-sensitive *E. coli* strains. These results suggest that these genes do encode efflux pumps and have critical roles in the biology of this soil-dwelling plant pathogen. This is the first report describing mechanisms underlying the intrinsic resistance of *R. solanacearum* to various toxic compounds, and to our knowledge, this is the first characterization of a member of the DinF subfamily of MATE MDRs in a phytopathogenic bacterium.

Plants produce a battery of antimicrobial compounds that function synergistically to limit pathogen colonization and spread. Indeed, inactivation of either *acrAB* or *dinF* resulted in reduced virulence and increased sensitivity to several antimicrobial compounds produced by plants, including caffeic acid, resorcinol, esculetin, tomatine, and berberine. However, the concentrations of the compounds tolerated were relatively high compared to the concentrations that inhibit animal or human pathogens (65). The difference may be explained by the discovery of a class of plant-derived compounds known as MDR inhibitors, which can significantly increase the antimicrobial properties of plant secondary metabolites (6, 62, 65). The sensitivity of *R. solanacearum* to these compounds in the presence of MDR inhibitors and to the full repertoire of toxins present in host plants, such as tomato, remains to be determined. The fact that both mutants were less tolerant to toxins that are produced by tomato (caffeic acid and tomatine), as

well as toxins that are produced by nonhost plants (resorcinol, esculetin, and berberine), implies that resistance to such toxins reflects a general bacterial defense response that is not limited to a particular host.

During the initial bacterial colonization, nutrients in the plant are scarce, especially in the xylem (49). In *E. coli*, *acrAB* expression increases in carbon-starved cells, probably because starvation triggers a general bacterial stress response, resulting in increased MDR expression (53). It is therefore not surprising that *R. solanacearum* *acrAB* expression also increases under carbon starvation conditions; *dinF* expression also increased slightly in the absence of carbon (data not shown).

An *R. solanacearum* global virulence gene regulator, PhcA, inhibits *acrAB* expression; as PhcA is expressed at higher cell densities (58), this correlates with our observation that *AcrA* expression generally decreases as cell density increases, at least in planta. Additionally, we found that the stress-responsive sigma factor RpoS was required for *acrAB* expression in minimal medium and when cell densities approached 10⁸ CFU/ml in planta, which is equivalent to the stationary phase. This finding is in contrast to *acrAB* expression in *E. coli*, which increases during the stationary phase and is not dependent on RpoS (53).

We found that the type III secretion regulator HrpB was required for maximal *dinF* expression at high cell densities in minimal medium, but at low cell densities in minimal medium and in planta loss of HrpB had no effect on *dinF* expression. This finding should be examined further because HrpB regulation of non-type III secretion genes is not well documented, even though the HrpB regulon is apparently large (16, 42).

Although *dinF* is not physically near genes known to be involved in either SOS repair or competence in *R. solanacearum*, we found that expression of *dinF* was induced by the DNA-damaging compounds mitomycin C and hydrogen peroxide at sublethal concentrations in culture (Table 5), even though there were not great differences in the MICs of these compounds (Tables 2 and 5). However, these compounds also induced expression of *acrAB*, which is not associated with the SOS response, suggesting that a general function of MDRs may be to expel DNA-damaging toxins from the bacterial cell, independent of a specific SOS response. Furthermore, such induction may reflect a general protective response to compounds that can potentially compromise the integrity of the bacterial DNA. This hypothesis is supported by the recent finding that in *E. coli* β -lactam antibiotics trigger the SOS response (39).

It has been hypothesized that polyphenols produced by the moderately resistant tomato cultivar H7996 might restrict pathogen spread through the root (68). Thus, we measured the expression of *acrAB* and *dinF* in H7996 and found that the expression of both *acrAB* and *dinF* was slightly induced at low cell densities but significantly reduced at high cell densities in this resistant cultivar compared to the expression in susceptible tomato plants. This finding was unexpected, but it is possible that high bacterial cell densities caused the resistant host to produce an as-yet-unknown factor that suppresses MDR expression.

The fact that *acrAB* and *dinF* are required for bacterial wilt virulence does not preclude the possibility that these pumps have an additional function in facilitating rhizosphere survival

and competition. The *E. amylovora* NorM efflux pump contributes to the ability of this bacterium to compete with other bacteria that occupy the same niche (11). MDRs may be required for fitness in rhizosphere environments containing plant root exudates and antimicrobial compounds produced by competitors. *R. solanacearum* is a successful inhabitant of tropical and subtropical soils, and the biology of its soil survival is poorly understood. Could the *acrAB*- and *dinF*-encoded MDRs in *R. solanacearum* also play a role in niche competition?

We found that loss of either of two *R. solanacearum* MDRs measurably increased sensitivity to various toxins. The moderate change in sensitivity likely reflected the presence of additional MDRs in the bacterium. Indeed, the *R. solanacearum* GMI1000 genome annotation suggests that there are at least 56 MDR genes, 32 of which appear to be organized in 12 separate operons. Five of these operons are located on the 3.7-Mb chromosome, and seven are on the 2.1-Mb megaplasmid (*acrAB* is on the chromosome, and *dinF* is on the megaplasmid). These genes include genes encoding MDRs belonging to the MATE superfamily (4), the RND superfamily (24), a group of *acrR*-type transcription regulators (9), the major facilitator superfamily (7), the ABC superfamily (4), and a group of unclassified drug efflux transporters (7). Only one gene encodes a protein that belongs to the SMR family; this gene was also identified in our recent *in vivo* expression technology screen, but it has not been studied yet. The presence of so many likely MDR-encoding genes throughout the genome implies that *R. solanacearum* must confront and export a wide range of toxic compounds, which may contribute to this pathogen's ability to infect such a broad range of plant hosts and survive saprophytically in the soil.

The contribution to virulence of MDRs in a nonhuman pathogen highlights the importance of MDRs in a natural (versus nosocomial) environment and indicates that these pumps increase bacterial fitness and are maintained by natural selection pressures. Furthermore, the study of MDRs in *R. solanacearum* can help us understand this pathogen's unusually wide plant host range and may suggest biocontrol measures. Additional research with this and other plant-bacterium systems will likely add to the accumulating body of evidence that plant- and microbe-derived toxins affect bacterial survival and virulence.

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